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**STUDY OF THE EFFECTS
OF 2,4,6-TRINITROTOLUENE (TNT) REACTIONS
ON PRODUCT MINERALIZATION BY ACTIVATED SLUDGE**

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PREFACE

The work described in this report was authorized under the Assembled Chemical Weapons Assessment Program. This work was started in April 1998 and completed in June 1998.

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STUDY OF THE EFFECTS OF 2,4,6-TRINITROTOLUENE (TNT) REACTIONS ON PRODUCT MINERALIZATION BY ACTIVATED SLUDGE

1. INTRODUCTION

Biodegradation of 2,4,6-trinitrotoluene (TNT) has been investigated under a variety of conditions. These include anaerobic sulfate-reducing bacteria that use TNT as their nitrogen source,¹ aerobic landfarming,² combined anaerobic/aerobic biodegradation using exogenous carbon sources,³ fungal degradation,⁴ composting,^{5,6} bacterial co-metabolism,⁷ genetic construction of a TNT-utilizing *Pseudomonas* strain,⁸ and enzymatic reduction.⁹ Important progress has been made in several of these areas, as reviewed by Rieger and Knackmuss,¹⁰ Bennett,¹¹ Kaplan,¹² and Spain.¹³ However, little success has been realized in the mineralization of TNT by activated sludge. Typically, TNT is reduced to recalcitrant products such as aminonitrotoluenes and azoxy compounds, with very little mineralization.¹⁴

Biodegradation offers the principal advantage of operating as a catalytic process. Aerobic mineralization occurs when enzymes catalyze the oxidation of organics to produce CO₂, a reaction that would not be favored abiotically at low temperatures. Biocatalysis greatly reduces the activation energy of degradative reactions, reducing the energy requirement and therefore the cost of waste treatment. Depending on the operating strategy, carbon, nitrogen and phosphorus can all be removed from wastewater. Microbial use of substrates as nutrient sources is important for process stability since it provides a selection. Microorganisms generally use carbon, nitrogen, phosphorus and sulfur, in that order of predominance.¹⁵ Therefore, utilization of organic substrates as carbon source is preferable since added nutrients will impact on the mass balance and cost of the process. With nitrogen-rich organics like TNT, microbial utilization of the substrate as both carbon and nitrogen sources is desirable, since nitrogen is also an important water pollutant.

Activated sludge is a versatile, naturally-selected microbial ecosystem, the reliability and economy of which are attested to by its widespread application in municipal and industrial treatment plants. However, sludge degrades TNT relatively poorly, accumulating dead-end metabolites, some of which are toxic.¹⁶ It is frequently assumed, although not proven, that essentially all naturally-produced compounds are biodegradable; the rationale for this assumption being that compounds would otherwise accumulate infinitely.¹⁷ However, xenobiotic (manmade) compounds such as TNT often prove recalcitrant to biodegradation. With some recalcitrant compounds, including many nitroaromatics, this may be due to their toxic and inhibitory effects on microorganisms.¹⁸ Other compounds may not be degraded simply because their relatively recent introduction into ecosystems has not allowed sufficient time for the evolution of the necessary degradative enzymes. The evolution or recruitment of degradative enzyme genes can sometimes be induced in the laboratory, as was done

with strain AC1100 which degrades 2,4,5-trichlorophenoxyacetic acid.¹⁹ In other cases, this apparently occurs in the natural environment, as evidenced by the evolution of atrazine-degrading enzymes from a common ancestor.²⁰ Also, recent results indicate that continuous culturing with selective pressures can markedly improve the ability of a mixed culture to mineralize TNT.²¹

One approach to make recalcitrant compounds biodegradable is to chemically alter the substrate to render it susceptible to biological attack. In the case of TNT treatment by activated sludge, the appropriate chemical pretreatment may offer a mechanism to bypass the formation of dead-end metabolites and permit mineralization to occur. Several reactions of TNT have been described in the literature, including those with base,²² reducing agents,²³ and ultraviolet (UV) light.²⁴

The primary intended application of TNT bioremediation studies to date has been the remediation of soil contaminated during munition loading and packing operations. A separate, but similarly significant need exists for TNT disposal technologies to support the demilitarization of chemical and conventional munitions. The United States has over 1.8 million chemical munitions stored at sites with no disposal facility.²⁵ A suitable disposal technology must address the energetic components as well as the chemical agents. Previous studies have addressed the chemical/biological processing of chemical agents.²⁶⁻²⁸ For demilitarization applications, a chemical pretreatment may convert TNT to biodegradable products, while simultaneously eliminating the explosive hazard. Coupled with a biological nutrient-based growth selection (such as sole carbon and/or nitrogen source utilization), this type of treatment might provide a stable and practical system for TNT disposal. The objective of this study was to investigate the relationship between various TNT reactions and mineralization of the resulting products as sole carbon and nitrogen sources by activated sludge.

2. MATERIALS AND METHODS

All chemical reactions were conducted overnight at 25° C in water with 100 mg/L TNT including 0.59 mg/ml of 21.6 mCi/mmol uniformly radiolabeled ring [¹⁴C]TNT (Chemsyn Science Laboratories, Lenexa, KS). Zinc powder, iron powder and NaBH₄ were used at 1000 mg/L and NaOH and HCl concentrations were 0.1 M. UV treatments were for 2 hr at 1 cm from a Fotodyne 3-3000 lightbox with a maximum output at 300 nm of about 0.12 μW/cm² @ 1.5 m. H₂O₂ concentrations were 3.4 mg/ml. Radioactivity was determined before and after each chemical reaction, and the recovery was consistently 97-100%, indicating no mineralization occurred during these reactions. The exception was the iron/HCl/H₂O₂ reaction, discussed below.

Activated sludge was aerated for 2-4 days prior to use to remove any residual biodegradable organics.

Scintillation counting was done in triplicate in a Beckman LS 6000IC counter with a counting efficiency of 95-97%.

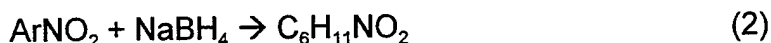
Nitrate levels were measured using the Hach (Loveland, CO) chromotropic acid method (#10020) with values determined spectrophotometrically at 410nm. Nitrite levels were measured using the Hach diazotization method (#8507).

3. RESULTS AND DISCUSSION

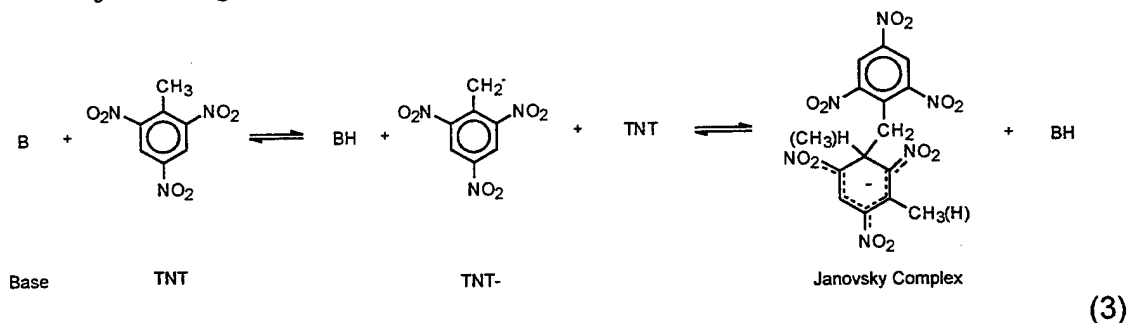
Initially, three reactions were studied: zinc/water, NaBH_4 and NaOH . Literature reports provide some information on these reactions. Nitroaromatics can react with water in the presence of zinc to form hydroxylamines:²⁹



NaBH_4 treatment has resulted in ring reduction to cyclohexane with nitro groups intact:³⁰



Strong base can deprotonate TNT to form the 2,4,6-trinitrobenzyl anion (TNT-) that can act as a nucleophile to attack another TNT molecule forming the Janovsky Complex, shown in Equation 3 with the position of nucleophilic attack unspecified, i.e., either $-\text{CH}_3$ or H might exist at either the 1 or 3 positions of the anionic ring.³¹



The first biodegradation experiment was a time course of mineralization with the pH-neutralized products of all three reactions and a TNT control. The products of each reaction were provided as sole carbon source to 1 mg/ml activated sludge in biometry flasks amended with inorganic nutrients only. CO_2 was trapped as carbonate in NaOH . No $^{14}\text{CO}_2$ was detected from sterile controls (not shown). Figure 1 shows that mineralization of untreated TNT was less than 1%, consistent with results seen by others.¹⁴ Mineralization of the reaction products was 1.3% with zinc, 5.0% with NaOH and 9.8% with NaBH_4 .

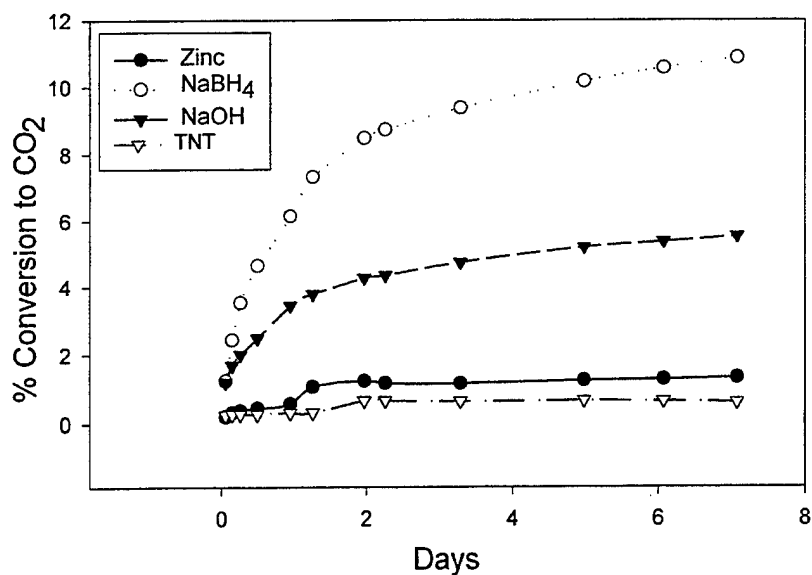


Figure 1. $^{14}\text{CO}_2$ release versus time for products of single reactions provided to activated sludge as sole carbon source.

Free nitrate and nitrite concentrations were measured in the products of each of the reactions and in the TNT control to determine if there was a relationship between mineralization and the extent of denitration. Figure 2 shows that these two values correlated very closely, presumably because aromatic nitro groups withdraw pi electrons from the aromatic nucleus and prevent electrophilic attack from oxygenases of aerobic organisms. Denitration therefore would facilitate mineralization. This is consistent with literature reports of mononitroaromatic versus polynitroaromatic biodegradation.¹⁸

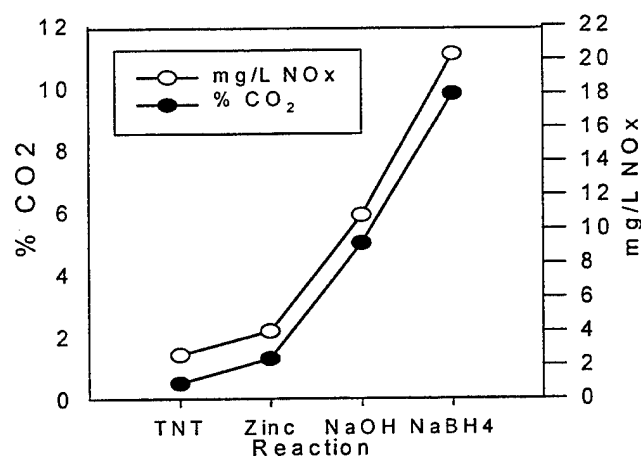


Figure 2. Correlation of denitration and mineralization for reactions shown in Figure 1.

In the second series of reactions, different combinations of zinc, NaOH, and NaBH₄ were tried in addition to other reactions incorporating iron, HCl, H₂O₂, and UV light. The products of all these reactions were provided to sludge as sole carbon and nitrogen sources for one week. Mass balances were completed by measuring the radioactivity present in the pellet and supernatant as well as the CO₂. Results are shown in Table 1. Reactions were either conducted sequentially (indicated as "then") or simultaneously (indicated by "+"). Although the pellets and supernatants were analyzed separately, the values were added and presented as one since the pellet can contain insoluble or adsorbed organics in addition to cells.

Table 1. Fate of various TNT reaction products

Chemical Reaction(s)	% ^{14}C as CO_2	% ^{14}C in pellet + supernatant	Total % ^{14}C recovered
Iron + HCl + H_2O_2	73.4	29.1	102.5
NaOH + H_2O_2	42.8	51.1	93.9
NaBH_4 , then NaOH	33.8	63.8	97.6
NaOH + Zinc	22.4	71.0	93.4
NaOH, then NaBH_4	18.8	75.1	93.9
NaOH + NaBH_4 + Zinc	16.7	80.4	97.1
Zinc + HCl	15.7	78.5	94.2
Zinc + UV light	14.9	78.7	93.7
NaBH_4 + Zinc	14.3	75.2	89.5
Iron + HCl	12.9	61.9	74.8
Iron + NaOH	12.2	81.9	94.2
NaBH_4 + H_2O_2	12.1	76.3	88.4
Iron + UV light	9.2	78.5	87.7
NaOH, then Zinc + HCl	7.0	76.7	83.6
NaBH_4 + HCl	1.7	87.1	88.8
Iron alone	3.8	73.0	76.8
TNT	0.9	85.9	86.8

4. CONCLUSIONS

Neither the optimal conditions and concentrations of reactants nor the practical utility of any of these reactions has yet been established. However, the mineralization data do provide insight into the relationship between TNT reactions and product biodegradability. First, all the reactions increase the mineralization of TNT as compared to the unreacted control. Second, there is a correlation between denitration and biodegradability. Since the TNT was uniformly labeled on the ring carbons only, all mineralization data pertain directly to the aromatic nucleus. Denitration of the aromatic ring appears to facilitate mineralization, presumably because the decreased electron withdrawal effects render the ring more susceptible to biological attack.

Among the different chemical reactions studied, the greatest mineralization was achieved with a combination of iron, HCl and H_2O_2 , which yielded a 73.4% conversion of reaction products to CO_2 . However, this reaction also mineralized approximately 37% of the TNT prior to biodegradation. The combination of NaOH and H_2O_2 permitted

42.8% conversion to CO_2 without any chemical mineralization. H_2O_2 is a very clean and convenient reagent since the only byproduct of its oxidizing reaction is water.

The data also indicate the importance of base reactions, either in combination with H_2O_2 , NaBH_4 , or with zinc. The order of reaction between NaBH_4 and NaOH is significant, since the mineralization level was almost twice as high when NaBH_4 preceded NaOH . In contrast, $\text{NaBH}_4 + \text{HCl}$ products were mineralized little more than unreacted TNT.

The effects of iron and HCl are of potential interest for mustard (2,2'-dichlorodiethyl sulfide) chemical munition disposal since significant quantities of both these materials are produced from the hydrolysis of munitions-grade mustard (Harvey et al., 1997a). When H_2O_2 was added to the iron/ HCl mixture, mineralization increased from 12.9% to 73.4%.

UV light, in combination with either iron or zinc, had only relatively moderate effects on TNT mineralization. Solutions turned red within minutes of initial exposure to the UV source, suggesting a rapid reaction. However, the two reactions yielded only 9.2% and 14.9% conversion to CO_2 , respectively.

Complete aerobic mineralization typically converts about 70% of the substrate to CO_2 and about 30% to new biomass (Slater and Somerville, 1979). Therefore, it would not be expected that more than approximately 70% of the starting material could be recovered as CO_2 , even with 100% recovery of the starting radioactivity. Inevitably, some sample loss occurs during transfer and processing. The seventeen experiments in Table 1 averaged 90.4% recovery.

These data comprise the first report of the mineralization of various TNT reaction products by activated sludge and provide insight into the basis of chemistry by which TNT can be rendered biodegradable.

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